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Extracellular vesicles released by CD40/IL-4 stimulated chronic lymphocytic leukemia cells  
confer altered functional properties to CD4<sup>+</sup> T cells

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## Key Points

- **CD40/IL-4 stimulated CLL cells release extracellular vesicles (EVs) enriched with specific miRNAs including miR-363.**
- **Transfer of CLL-EVs to autologous CD4<sup>+</sup> T cells enhances migration and immune synapse formation interactions with tumor cells.**

## Abstract

The complex interplay between cancer cells, stromal cells and immune cells in the tumor microenvironment (TME) regulates tumorigenesis and provides emerging targets for immunotherapies. Crosstalk between CD4<sup>+</sup> T cells and proliferating chronic lymphocytic leukemia (CLL) tumor B cells occurs within lymphoid tissue pseudo-follicles and investigating these interactions is essential to understand both disease pathogenesis and the effects of immunotherapy. Tumor-derived extracellular vesicle (EV) shedding is emerging as an important mode of intercellular communication in the TME. In order to characterize tumor EVs released in response to T cell-derived TME signals, we performed microRNA (miR) profiling of EVs released from CLL cells stimulated with CD40 and IL-4. Our results reveal an enrichment of specific cellular miRNAs including miR-363 within EVs derived from CD40/IL-4 stimulated CLL cells compared to parental cell miRNA content and control EVs from unstimulated CLL cells. We demonstrate that autologous patient CD4<sup>+</sup> T cells internalize CLL-EVs containing miR-363 that targets the immunomodulatory molecule CD69. We further reveal that autologous CD4<sup>+</sup> T cells that are exposed to EVs from CD40/IL-4 stimulated CLL cells exhibit enhanced migration, immunological synapse signaling and interactions with tumor cells. Knockdown of miR-363 in CLL cells prior to CD40/IL-4 stimulation, prevented the ability of CLL-EVs to induce increased synapse signaling and confer altered functional properties to CD4<sup>+</sup> T cells. Taken together, these data reveal a novel role for CLL-EVs in modifying T cell function that highlights unanticipated complexity of intercellular communication that may have implications for bidirectional CD4<sup>+</sup> T-cell:tumor interactions within the TME.

## Introduction

Chronic lymphocytic leukemia (CLL) is one of the most common adult B cell malignancies and is characterized by the clonal expansion of CD5<sup>+</sup> mature B-cells in lymph nodes and the peripheral blood.<sup>1,2</sup> Emerging research suggests that the complex interplay between leukemic cells and stromal cells, including immune cells, in the lymphoid tissue tumor microenvironment (TME) critically regulates tumor progression and the licensing of immune evasion.<sup>3-6</sup>

CLL tumor cells have been shown to evade proximal anti-tumor T cell activity by delivery of inhibitory signals (including via the PD-L1:PD-1 immune checkpoint pathway)<sup>6</sup> into T cells to down-regulate lytic immune synapse activity. However, proliferating CLL cells co-localize with CD4<sup>+</sup> T-cells within lymph node pseudo-follicles (proliferation centres) suggesting intercellular activatory signaling and tumor-promoting interactions.<sup>7</sup> Understanding tumor-T cell crosstalk will be essential to overcome complex pro-tumorigenic immune regulatory mechanisms in the TME. Moreover, biological insight into tumor:CD4<sup>+</sup> T-cells communication will help understand the effect of activating T cells in the CLL TME using novel immune checkpoint immunotherapy such as anti-PD-1 antibody drugs.<sup>8-10</sup>

Although cell-to-cell contact (ligand:receptor interactions) and exchange of soluble factors (classical paracrine growth factor signalling loops) are key mediators of intercellular communication within the TME, more recently, extracellular vesicle (EV) shedding has emerged as another mode of cell-cell signalling.<sup>11</sup> EVs comprise exosomes (30 - 100 nm), which have an endocytic origin and larger microvesicles, which are released from the plasma membrane. Categorizing vesicles based on their size or subcellular origin remains problematic<sup>12</sup> and whether or not, one type of vesicle is biologically more relevant than another is currently unclear within the field.<sup>13</sup>

Transfer of EVs and their cargo, which includes proteins, lipids, mRNA and miRNAs, from cancer cells to other TME cell types has been the subject of intense study in the solid cancers<sup>13</sup> but relatively understudied in lymphoid malignancies. Recent work has revealed that B cell receptor (BCR) activation, a critical survival pathway within the CLL TME, enhances secretion of CLL exosomes that carry a distinct microRNA (miRNA) signature compared to healthy donors.<sup>14</sup> Moreover, CLL EVs and their molecular cargo are actively transferred to stromal cells inducing intracellular signaling and the reprogramming of these cells into proinflammatory cancer-associated fibroblasts (CAFs).<sup>5,15,16</sup>

The release of EVs is enhanced when primary murine B cells are stimulated by T cell signals<sup>17</sup> but the effects of CD40 and IL-4 stimulation, amongst the most important immune-derived TME

stimuli,<sup>18,19</sup> on the production and content of CLL-EVs has not previously been investigated. Here, we determine those miRNA selectively enriched or depleted in CLL-EVs following CD40/IL-4 stimulation (CD40/IL-4 CLL-EVs). We show that miR-363 is highly enriched in CD40/IL-4 CLL-EVs and that a principal target for this miRNA is the T cell immunoregulatory receptor CD69. Critically, we show that the transfer of CLL-EVs into autologous CD4<sup>+</sup> T cells increases motility rate, immunological synapse signaling and interactions with tumor cells. These results define a novel immunomodulatory mechanism in CLL induced by EVs and their molecule cargo, which might have important implications for crosstalk between the TME and CLL cells.

## **Materials and methods**

### **Patient Samples**

Mononuclear cells were isolated from heparinised whole blood from CLL patients (local research ethics committee approval with informed consent), using Histopaque density gradient media (Sigma, St. Louis, MO, USA) with density gradient centrifugation. CD19<sup>+</sup> CD5<sup>+</sup> CLL were purified using negative selection for the EV isolation studies (MACS system; Miltenyi Biotec). Patient characteristics are presented in **Supplemental Table 1**.

### **EV isolation**

CD19<sup>+</sup> CLL cells were cultured in Aim V with AlbuMAX serum free media (Invitrogen, Paisley, Scotland) at a concentration of  $3 \times 10^6$  /ml. To stimulate the release of CLL B-cell EVs, CLL cells were incubated with recombinant human CD40 ligand (sCD154) (1 µg/ml) (R&D Systems, Lexington, MA, USA) and IL-4 (20 ng/ml) (R&D Systems); and anti-CD40 antibody (clone EA-5) at 1 µg/ml (Santa Cruz Biotechnology, Santa Cruz, CA, USA; sc65264) for 36 hours. CLL-EVs were harvested from  $36 \times 10^6$  cultured leukaemic cells at 4°C by differential centrifugation of culture supernatant. Supernatants were spun at 250 g for 5 minutes followed by 2000 g for 10 minutes to pellet cells and debris; and 10 000 g for 30 minutes to pellet debris and larger membrane bound particles. EVs were pelleted by centrifugation at 100 000 g for 110 minutes, washed in PBS then re-pelleted, and finally re-suspended in culture media for analysis (see supplemental Methods for details).<sup>14</sup>

### **MiRNA microarrays**

MiRNA extracts were made using a miRvana miRNA isolation kit (Applied Biosystems, Paisley, UK). RNA concentration was measured (NanoDrop 1000 Spectrophotometer, Thermo Fisher Scientific, Wilmington, DE, USA) and equal amounts were reverse transcribed using MegaPlex RT Primers and a TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems). Equal volumes were loaded on human microRNA Array (A) plates (containing assays for 377 miRNA) (Applied Biosystems, #4398965) and reactions carried out on a 7900HT Real Time PCR system.<sup>20</sup> 40 PCR cycles were carried out and C<sub>t</sub> values >35 were not included in the analysis. All C<sub>t</sub> values were normalized to the global plate mean of expressed miRNA C<sub>t</sub> values, and ΔC<sub>t</sub>

values were calculated. In order that each plate had approximately the same mean Ct value both cellular and extracellular vesicle samples loaded on the same plate. The microarray data has been deposited with GEO, accession number GSE54434.

### **CLL cell transfection**

CLL cells were cultured in the presence or absence of CD40/IL-4 stimulation in 6-well plates at  $1 \times 10^7$ /ml with a volume of 2 ml/well. Either miRCURY LNA Power microRNA inhibitor miR-363 (Exiqon, catalogue number 4101050-101) or miRCURY inhibitor negative control A (Exiqon, catalogue number 199006-101) were added to the cultures at a final concentration of 50 nM for 48 hours before harvesting. In order to knockdown Rab27A and Rab27B anti-sense LNA GapmeRs (Exiqon, design ID 554767-1, catalogue number 300602-00) were employed at 100 nM for 96 hours. Negative control anti-sense LNA GapmeR (Exiqon, catalogue number: 300612-00) was also employed. Cells were harvested and degree of knockdown checked by western with anti-Rab27 antibody (Proteintech, Manchester, UK, catalogue number 17817-AP) at 1:1000.

### **Immune synapse assay.**

Immune synapse assays were performed as previously described (see supplemental Methods for details).<sup>6,21</sup>

### **Motility video microscopy**

The Motility studies were performed and analyzed as previously described (see supplemental Methods for details).<sup>22</sup>

### **Statistical methods**

Statistical methods used in this study are outlined within the respective methodology sections (see supplemental Methods for details) and Figure Legends. Data were compared with the use of either the 2-tailed Student *t* test or using the nonparametric Mann-Whitney test. Analysis was done using Prism Version 5 software. *P* < .05 was considered statistically significant.

## **Results**

### **Characterization and miRNA profiling of CLL-EVs derived from CD40/IL-4-stimulated CLL cells reveals enrichment of specific miRNA content including miR-363**

EVs from patient plasma (n=23) were compared to age-matched healthy donors (n= 10) by dynamic light scattering (NanoSight, Malvern Instruments). Although the distribution of vesicle sizes was similar (**Figure 1A**), CLL patients showed significantly (*P* = 0.03) increased numbers of EVs compared to healthy donors (**Figure 1B**). The EV particle size distributions for CLL cases with higher or lower numbers of plasma vesicles were similar (**Figure 1C**) and EV concentration was not significantly correlated with absolute lymphocyte or platelet counts

(**Figure 1D**). These findings are in agreement with previous studies<sup>14,15</sup> and suggest that EV concentration in CLL does not simply reflect circulating tumor load.

To examine the contribution of immune-derived CD40 and IL-4 stimulation we characterized EVs released from CLL cells following CD40 and IL-4 (CD40/IL-4 CLL-EVs).<sup>18,19</sup> CD40/IL-4 stimulation provide signals for leukemia survival,<sup>19</sup> in part through induction of anti-apoptotic proteins and proliferation.<sup>23</sup> To avoid confounding effects due to EVs produced by a feeder cell layer, we utilised soluble stimulators: anti-CD40 antibody, soluble CD40L and IL-4<sup>18</sup> (**Supplemental Figure 1A-C**). Unstimulated CLL-EVs (mean 134 nm, mode 110 nm) and CD40/IL-4 CLL-EVs (mean 150 nm, mode 100 nm) showed similar particle concentration and size distribution (**Supplemental Figure 1D and 1E**). Although the CLL leukemia cell line MEC1 produced ~15-fold more EVs (**Supplemental Figure 1F**) compared to primary CLL cells, their particle size (mean 120 nm, mode 91 nm) was comparable to EVs from primary tumor cells.<sup>5</sup> The tetraspanin protein CD9 was highly expressed on parental CLL cells and was detectable on CD40/IL-4 CLL-EVs, while CD63 was induced on both parental tumor cells and CLL-EVs following CD40/IL-4 stimulation (**Supplemental Figure 1G and H**).

Next we employed low-density microarrays (**Figure 1E and F**) to compare miRNA profiles between EVs derived from CD40/IL-4 stimulated CLL cells and those of unstimulated parental cells (CLL). We found EV miRNA content to be less complex than that of cells<sup>5,24</sup> (19 miRNAs in EVs compared to 136 in CLL cells). A signature of 13 ( $FDR < 0.05$ ) or 7 ( $FDR < 0.01$ ) miRNA that were differentially expressed between EV and cells was obtained: miR-363, miR-374b, miR-323-3p and miR-494 were up-regulated and miR-150, miR-484 and miR-17 were down-regulated (**Figure 1F and G**). Ranking miRNA from the most highly to the least expressed showed that individual miRNA that were at relatively low levels in cells could be more highly expressed within EVs, indicating selective inclusion of miRNAs into EVs from the cellular pool (**Figure 1H,I and Supplemental Table 2**). We validated the microarray findings by comparative RT-PCR analyses of paired EVs and parental CLL cells with and without CD40/IL-4 stimulation (**Supplemental Figure 2**). Enrichment of miR-363, miR-374b, miR-323-3p and miR-494 in EVs compared to parental CLL cells was confirmed (**Supplemental Figure 2A**). Although the amounts of these miRNA did not differ between unstimulated and stimulated parental CLL cells (**Supplemental Figure 2B**), we detected significant enrichment of miR-363, miR-374b, miR-323-3p and miR-494 in EVs derived from CD40/IL-4 stimulated CLL cells (CD40/IL-4 EVs) compared to EVs from unstimulated cells (CLL-EVs) (**Supplemental Figure 2C**). Although some of the differentially expressed EV miRNAs (miR-155, miR-150, miR-191 and miR-223) identified from our miRNA profiling have been associated with CLL plasma-derived exosomes and BCR activation,<sup>14</sup> or from cultured CLL cell-derived exosomes,<sup>16</sup> our data shows that CD40/IL-4 stimulation contributes to enrichment of specific miRNA within CLL-EVs.

#### **CLL-EVs carrying miR-363 can be functionally transferred to target CD4<sup>+</sup> T-cells**

MiR-363 showed ~270-fold increased expression in EVs compared to parental CD40/IL-4 stimulated CLL cells (**Supplemental Table 2**) and we identified the T cell immunomodulatory

protein, CD69<sup>25</sup> as having the highest context score of all miR-363 target genes ([targets.can.org](http://targets.can.org))<sup>26,27</sup> (**Supplemental Table 3**). Three potential conserved miR-363 binding sites are present in the CD69 3' UTR (**Figure 2A-C**) and luciferase reporter assays were carried out to confirm their functional role. Transfection of a miR-363 mimic significantly repressed CD69 luciferase activity, while mutation of all three miR-363 binding sites was required to maximally abolish repression of luciferase activity by miR-363 (**Figure 2D** and **Supplemental Table 4**).

To show functional importance of miR-363 we transfected Jurkat T cells with either a miR-363 mimic or a scrambled control miRNA prior to PMA/ionomycin stimulation to induce CD69 expression. RT-PCR for miR-363 demonstrated increased levels within Jurkat T cells following transfection (**Supplemental Figure 3A**) and this associated with significantly repressed PMA/ionomycin-induced expression of CD69 ( $P < .05$ ) (**Figure 2E**). CD69 expression was inducible on primary CD4<sup>+</sup> T cells following cross-linking with anti-CD3/CD28. Next, primary CD4<sup>+</sup> T cells were cultured with CLL-EVs following CD40/IL-4 stimulation (CD40/IL-4 CLL-EVs) compared to control EVs from unstimulated CLL cells (CLL-EVs) in order to determine their effect on CD69 expression. CD40/IL-4 CLL-EVs increased expression levels of miR-363 in target T cells ( $P < 0.01$ ) (**Figure 2F**) and led to significant suppression of CD69 expression following cross-linking ( $P < .05$ ) (**Figure 2G**). To confirm uptake of CLL-EVs by primary T cells transfer co-culture experiments were carried out and demonstrated maximal uptake of fluorescently (PKH67) labeled CD40/IL-4 CLL-EVs into autologous CD4<sup>+</sup> T-cells by 24 hours (**Figure 3A-C**). Taken together, we show that EVs released from CD40/IL-4 stimulated CLL cells that carry miR-363 can be functionally transferred to target CD4<sup>+</sup> T-cells, resulting in direct modulation of the mRNA target CD69.

#### **Autologous CD4<sup>+</sup> T cells exposed to EVs from CD40/IL-4 stimulated CLL cells exhibit enhanced immunological synapse signaling and migration.**

Given the link between CLL cells and CD4<sup>+</sup> T-cells within TME proliferation centres,<sup>7,19</sup> we hypothesized that EVs from CD40/IL-4 stimulated CLL cells might influence T cell function. Treatment of autologous CD4<sup>+</sup> T cells with CD40/IL-4 CLL-EVs significantly enhanced F-actin immunological synapse formation with CLL tumor cells compared to control CLL-EVs ( $P < .01$ ) (**Figure 4A**). Transwell culture assays confirmed that the transfer of secreted particles from CD40/IL-4-stimulated CLL cells modulated T cell immune synapse function compared to unstimulated CLL cells (data not shown). In addition, we observed that the percentage efficiency of CD4<sup>+</sup> T-cells to form conjugates was increased ( $P < .05$ ) following exposure to CD40/IL-4 CLL-EVs compared to control CLL-EVs (**Figure 4B**). Next, we examined the recruitment/polarization of proximal T cell receptor (TCR)-mediated tyrosine kinases to CD4<sup>+</sup> T-cell immune synapses following treatment with CLL-EVs. Our results showed significantly increased recruitment of Lck ( $P < .01$ ) (**Figure 4C**) and tyrosine-phosphorylated proteins (data not shown) to the T cell:CLL synapse contact sites following exposure to CD40/IL-4 CLL-EVs compared to CLL-EVs. Notably, treatment of CD4<sup>+</sup> T cells with CD40/IL-4 CLL-EVs augmented immune synapse activity while down-regulating CD69 expression levels on T cells compared to



CLL-EVs ( $P < 0.01$ ) (**Figure 4D**). To validate the ability of CD40/IL-4 CLL-EVs to modulate T cell synapse signaling, we knocked down Rab27A/B in parental CLL cells using locked nucleic acid (LNA)-based antisense oligonucleotides (**Supplemental Figure 4A**) prior to CD40/IL-4 stimulation and isolation of released EVs. Rab27A/B participates in the traffic of EVs including exosomes to the cell periphery as part of the secretory pathway.<sup>11</sup> We found that Rab27A/B knockdown in CLL cells impaired the release of EVs (**Supplemental Figure 4B**), which prevented modulation of synapse signalling (**Supplemental Figure 4C**). CD40/IL-4 CLL-EV-mediated increased synapse formation with enhanced early CD4<sup>+</sup> T cell signaling would be expected to augment T cell proliferation. To investigate this, we measured nuclear Ki-67 expression, a marker for cellular proliferation, in autologous patient CD4<sup>+</sup> T cells exposed to both CLL-EVs and anti-CD3/-CD28 crosslinking. As shown in **Figure 4E-G**, co-culture with CD40/IL-4 CLL-EVs increased Ki-67 expression in CD4<sup>+</sup> T cells compared with co-culture with control CLL-EVs. CFSE-labeled CD4<sup>+</sup> T cells confirmed that exposure to CD40/IL-4 CLL-EVs enhanced T cell proliferation (data not shown).

Rapid motility is essential for the trafficking of T cells into lymphoid tissue.<sup>28</sup> Video microscopy with tracking analysis was then used to assess the migration of CLL-EV-treated autologous CD4<sup>+</sup> T cells on ICAM-1 coated plates using the chemokine CCL19, which attracts T cells within the lymph node.<sup>22,28</sup> Our data show that treatment of CD4<sup>+</sup> T-cells with CD40/IL-4 CLL-EVs significantly increased migration rates (both speed and distance) compared with control CLL-EV treated T cells (paired t-test,  $P < 0.01$ ) (**Figure 5A-C** and **Supplemental Videos 1 and 2**, respectively).

#### **Knockdown of miR-363 in CLL cells prevents the ability of released CLL-EVs to confer altered functional properties to CD4<sup>+</sup> T cells**

To determine whether CLL-EV miR-363 is a significant regulator of CD4<sup>+</sup> T cell biology, we knocked down expression of this miR in CLL cells using locked nucleic acid (LNA)-based antisense oligonucleotides, prior to CD40/IL-4 stimulation and isolation of released EVs (**Supplemental Figure 3B**). MiR-363 knockdown prevented the ability of CD40/IL-4 CLL-EVs to confer increased F-actin immune synapse polarization at CD4<sup>+</sup> T cell contact sites with autologous CLL tumor cells (**Figure 6A**). Moreover, miR-363 knockdown prevented enhanced tyrosine kinase recruitment to the synapse site (**Figure 6B**) compared to CD40/IL-4 CLL-EVs derived from CLL transfected with scrambled control antisense oligonucleotides. Critically, CD69 expression levels remained unaffected in CD4<sup>+</sup> T cells following exposure to CD40/IL-4 CLL-EVs harvested from miR-363 knocked down CLL cells (**Figure 6C**), providing further evidence that the immunomodulatory receptor CD69 is a target of CD40/IL-4 CLL-EV miR-363.

We further show that treatment of autologous CD4<sup>+</sup> T cells with CD40/IL-4 CLL-EVs derived from miR-363 knocked down cells blocked modulation of T cell migration rates when compared to CD40/IL-4 CLL-EVs derived from control transfected tumor cells (**Figure 6D,E** and **Supplemental Videos 3 and 4**, respectively). These data together identify the capacity of

CD40/IL-4 CLL-EV miR-363 to play a critical role in regulating T cell motility and immune synapse signaling function.

## Discussion

Complex bidirectional crosstalk between CLL tumor cells and non-malignant cells within the TME plays a critical role in activating tumor survival, proliferation and fostering immune privilege.<sup>3,29</sup> T cells from CLL patients exhibit impaired F-actin immunological synapse formation that contributes to reduced activation and effector functions.<sup>21</sup> CLL tumor cells can suppress proximal activated anti-tumor T cell activity by delivering inhibitory signals via co-option of the immune checkpoint network including the PD-L1:PD-1 pathway to dampen formation of the lytic immune synapse.<sup>6</sup> Immune checkpoint blockade immunotherapy (including anti-PD-1 and anti-PD-L1 antibody drugs) has the potential to activate T cells and unleash anti-tumor immune responses, serving as an illustrative example of therapeutically targeting/re-educating the TME. However, there is evidence that subverted CD4<sup>+</sup> T cell subsets within the lymphoid TME may exhibit tumor-promoting activity that complicates our understanding of cancer immunology and raises the issue that immunotherapy might have negative as well as positive activity.<sup>4</sup> Investigating multifaceted bidirectional tumor-T cell crosstalk will be essential to understand both disease pathogenesis and the effects of novel immunotherapies within the TME.

CLL cells proliferate in distinct microanatomical tissue sites termed proliferation centers (pseudofollicles) that allow intimate interactions with TME components including activated CD4<sup>+</sup> T cells.<sup>1</sup> Immunohistochemistry analysis demonstrated that CLL cells in proliferation centers are in close proximity to CD4<sup>+</sup> T cells.<sup>30</sup> More recently, imaging studies have revealed that proliferating CLL cells were found to co-localize and form intercellular conjugates with activated CD4<sup>+</sup> T cells in lymph node tissue.<sup>7</sup> Activated CD4<sup>+</sup> T cells including follicular T helper cells (T<sub>FH</sub>) cells can provide CD40L co-stimulation and are a source of pro-survival cytokines (IL-4 and IL-21) that activate ERK, STAT and NF-κB signaling and CLL proliferation.<sup>31-34</sup> Moreover, a key role for CD4<sup>+</sup> T cells in promoting CLL cell survival and expansion has been identified using a humanized model of disease. Activated CD4<sup>+</sup> T cells were found to localize in pseudo-follicular structures (proliferation center-like) and surround CLL tumor cells within murine lymphoid TME tissue.<sup>35</sup>

Among the mechanisms by which cells communicate within the TME, emerging evidence suggests that EVs released from tumor cells have the ability to modulate the microenvironment to promote immune evasion, angiogenesis and stromal cell activation.<sup>13,36</sup> It is believed that EVs have an important role in intercellular communication as they have shown the potential to transfer their contents including proteins, lipids and RNAs (including mRNA and miRNA) between cells.<sup>13,37</sup> Early studies focussed on the ability of cancer cell-derived EVs that express tumor antigens to activate DCs as a means to elicit anti-tumor immunity. However, there is now mounting evidence suggesting predominantly immunosuppressive functions for tumor EVs<sup>13</sup> including the induction of T cell apoptosis,<sup>38</sup> suppression of TCR signaling and promoting the

activity of T regulatory cells ( $T_{\text{regs}}$ )<sup>39</sup> or tolerogenic DCs.<sup>40</sup>

MiRNAs (miRs) are important components of the molecular cargo of EVs and recent work has identified a disease-relevant EV microRNA profile in CLL.<sup>14,16</sup> In particular, work has revealed the influence BCR signaling has on the release of CLL EVs and the inclusion of selective miR content including miR-150 and miR-155.<sup>14</sup> The potential of CLL cell-derived EVs to modulate and re-educate the host microenvironment has been demonstrated with studies showing that CLL-EVs and their molecular cargo can be actively transferred to stromal cells inducing intracellular signaling and the reprogramming of these cells into proinflammatory cancer-associated fibroblast-like cells.<sup>5,15</sup> However, the effects of CLL-EVs on T cells have not been previously described.

In the present study, we aimed to characterize EVs released from primary human CLL cells in response to the T cell-derived TME signals CD40 and IL-4. These extracellular stimuli are known to produce prosurvival and proliferative signals in CLL.<sup>23,41</sup> We report on the miR profiling of CLL-EVs derived from CLL cells stimulated with CD40 and IL-4 and reveal an enrichment of specific cellular miRNAs including miR-363 compared to parental cell miRNA content and control EVs from unstimulated CLL cells. Our data shows that CD40/IL-4 stimulation contributes to the inclusion of selective miRNA within CLL-EVs distinct from BCR activation<sup>5,14</sup> or cultured CLL cell-derived exosomes.<sup>16</sup>

We demonstrate that autologous patient  $CD4^+$  T cells internalize CLL-EVs containing miR-363 that targets the immunomodulatory receptor CD69. Our data supports the functional transfer of this miR to target  $CD4^+$  T-cells. Bioinformatic analysis revealed that CD69 was a target gene for miR-363, one of the most highly enriched miRNA in CLL-EVs released from CD40/IL-4-stimulated CLL cells (~270-fold increased expression in EVs compared to parental CLL cells). CD69 is an early T cell activation molecule expressed at sites of chronic inflammation.<sup>25</sup> The precise immunomodulatory function of CD69 has not been elucidated owing to the absence of a known ligand and adequate *in vivo* models to study its physiological function. Although early data suggests that CD69 acts as a co-stimulatory receptor, recent studies have suggested a more complex non-redundant role for this receptor in the down-regulation of T cell responses<sup>25,42</sup> including inhibiting the migration of effector T cells.<sup>43,44</sup>

Our functional studies reveal that exposure of  $CD4^+$  T cells to EVs from CD40/IL-4 stimulated CLL cells enhances their migration, immunological synapse signaling, interactions with autologous tumor cells and proliferation. Immune synapses are cellular signaling structures that orchestrate the complex communication between the T cell and an antigen-presenting cell (APC) in a way that ensures detailed antigen recognition and effective T responses<sup>22</sup> (and potentially bidirectional cooperative activation signaling to B cells)<sup>45</sup>. Notably, treatment of  $CD4^+$  T cells with CLL-EVs augmented immune synapse activity while down-regulating CD69 expression levels on T cells. Intriguingly, knockdown of miR-363 in CLL cells using LNA antisense oligonucleotides prior to CD40/IL-4 stimulation, prevented the ability of CLL-EVs to induce increased synapse signaling and confer altered functional properties to  $CD4^+$  T cells,

suggesting an unsuspected and important role for this specific miRNA in regulating T cell function. CD69 expression levels remained unaffected in these CD4<sup>+</sup> T cells exposed to miR-363 knocked down CLL-EVs compared to control antisense oligonucleotides, providing evidence that T cell CD69 is a target of transferred EV miR-363. This data supports an important role for CD40/IL-4 CLL-EV-derived miR-363 in modulating CD4<sup>+</sup> T cell activity. In addition, these findings link the regulation of the immunomodulatory receptor CD69 to the miRNA cargo of CLL-EVs and suggest that CD69 may have a potential negative regulator function in CLL patient CD4<sup>+</sup> T cells. Clearly, future studies will need to determine the precise roles of miR-363 and CD69 in generating the EV-induced T cell functional effects we have observed. MiR-363 levels in CLL patients' plasma correlates with disease stage and some prognostic factors.<sup>46</sup> Together with our data, this supports a rationale for further studies investigating the potential of miR-363 to act as a TME-derived biomarker for disease biology and response to therapy including immunotherapy. It is likely that miR-363 is not the only miRNA capable of repressing CD69 and indeed miR-374b, which we also found to be enriched in our CLL-EVs, has a potential binding site at bases 26 to 32 in the 3' UTR of CD69.

Taken together, these data reveal a novel role for CLL-EVs in modifying CD4<sup>+</sup> T cell function that highlights unanticipated complexity of intercellular communication within the TME. The capacity of CLL-EVs and their specific molecular cargo (released following the T cell-derived signals CD40 and IL-4) to enhance CD4<sup>+</sup> T cell migration and immune synapse interactions with autologous tumor cells may represent a novel immune regulatory mechanism in the CLL TME.

### **Acknowledgments**

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### **Conflict of Interest**

The authors declare no conflict of interest.

### **Author contribution**

DTS, SW, BA, AGR and SDW designed experiments. DTS, BA, SW, LL, AA, ARA and GDR carried out experiments and analyzed data and AGR and SDW supervised the experiments, analyzed data and wrote the manuscript.

## Figure Legends

### Figure 1. EVs from CD40/IL-4 stimulated CLL cells are enriched for specific miRNAs.

Dynamic light scattering (NanoSight) was utilised to compare (A) EV size between plasma of patients (CLL) (n=23) and age-matched healthy donors (HD) (n=10) (n.s. = not significant) and (B) EV particle number/concentration. EV numbers were significantly greater in CLL patients compared to HDs (Mann-Whitney test;  $P=0.03$ ). Horizontal bars represent median values. (C) Comparison of EV size distribution in two patients; one with high EV particle number (solid line) and the other with lower EV particle numbers (dashed line). (D) Lack of association between total white cell count (WCC, black circles) or platelet count (PLT, grey circles) and CLL-EV numbers. (E) Electron micrographs of CLL-EVs obtained after differential centrifugation of supernatant from CLL cells treated with anti-CD40 antibody (clone EA-5, 1 $\mu$ g/ml), soluble CD40L (1  $\mu$ g/ml) and IL-4 (20 ng/ml) for 36 hours. Upper and lower panels show representative CLL-EV particles obtained from two individual CLL patients. The size marker is 100 nm. (F) Unsupervised hierarchical clustering of miRNAs with significantly different expression between CLL-EVs and paired parental intracellular fractions from 8 CLL patients following CD40/IL-4 stimulation (Wilcoxon, Mann-Whitney test utilising an FDR significance criterion limit of 0.05). (G) MiRNAs that showed significant differences (Wilcoxon, Mann-Whitney test utilising an FDR significance criterion limit of 0.01) in expression between CLL-EVs and paired parental intracellular fractions following CD40/IL-4 stimulation. Data is presented using box and whisker plots. MiR-363, miR-374b, miR-323-3p and miR-494 demonstrate increased expression in CLL-EVs whereas miR-150, miR-484 and miR-17 show reduced expression compared to parental CLL intracellular miRNA content. (H) Relative expression analysis plot. Cellular miRNA were ranked from the most highly expressed (relative expression arbitrarily set at 1) to the least expressed. The positions of the 4 miRNA whose expression level increased in CLL-EVs compared to parental CLL intracellular miRNA content is shown by the red arrows. The analysis shows that miRNAs that are highly expressed in CLL-EVs are a specific population and do not simply reflect the most highly expressed parental cellular miRNA content. (I) Pie charts showing relative distribution of miRNAs in parental CLL cells and paired CLL-EVs following CD40/IL-4 stimulation. The most highly expressed miRNAs in each compartment are labeled.

### Figure 2. Transfer of CLL-EVs containing miR-363 to CD4<sup>+</sup> T cells downregulates the expression of the immunomodulatory receptor CD69.

(A) Potential binding sites for miR-363 in the 3'UTR of human CD69 identified by TargetScan v7.0. (B) Diagram showing the position of the three miR-363 binding sites (labelled 1, 2 and 3) (TargetScan v7.0) in the CD69 3' UTR. The 3' UTR has a total length of 995 bp and site 1 is at 758 bp, site 2 at 870 bp and site 3 at 907 bp. (C) Plots demonstrating conservation of miR-363 binding sites (horizontal black lines). Sequences from six species (human, mouse, dog, cat, cow and rabbit) were compared (weblogo.berkeley.edu). The overall height of the stack indicates the sequence conservation at that position, while the height of symbols within the stack indicates the relative frequency of

each amino or nucleic acid at that position. **(D)** Luciferase activity, in HEK293T cells, following co-transfection of reporter constructs with either miR-363 mimic (black columns) or scrambled control miRNA (white columns) expressed as percentage of luciferase activity following transfection of the reporter construct alone. Five reporter constructs were tested; wild-type (WT), construct bearing mutations of site 1 (labeled 1), construct bearing mutations of site 2 (labeled 2), construct bearing mutations of site 3 (labeled 3) and construct bearing mutations of sites 1, 2 and 3 (labelled 1,2,3). The miR-363 mimic caused repression of luciferase activity compared to the scrambled control unless all three miR-363 sites were mutated ( $n=4$ ). Mean  $\pm$  SEM. The 2-tailed Student  $t$  test was used ( $*** P<0.001$ ,  $** P<0.01$ ,  $* P<0.05$  and n.s. not significant). **(E)** Flow cytometry histograms showing repression of CD69 expression in the human Jurkat T cell following transfection with miR-363 mimic. T cells were either transfected with scrambled control miRNA (solid blue line) or miR-363 mimic (solid red line) and stimulated with PMA/ionomycin. FACS histograms for control unstimulated cells are shown as red and blue dotted lines. Bar chart shows that miR-363 mimic caused a significant (Student  $t$  test,  $* P<0.05$ ) reduction in mean fluorescence intensity (MFI) ( $n=4$ ). **(F)** Incubation of primary CD4<sup>+</sup> T-cells with autologous CLL-EVs following CD40/IL-4 stimulation increased intra-cellular levels (relative change) of T cell miR-363. Mean  $\pm$  S.E.M. from 3 separate experiments are shown. (Student  $t$  test,  $** P<0.01$ ) **(G)** Flow cytometry analysis revealing that incubation of primary CD4<sup>+</sup> T cells with autologous CLL-EVs following CD40/IL-4 stimulation (red line) led to significant suppression of CD69 expression following cross-linking of T cells with anti-CD3/CD28 compared to the treatment of T cells with control CLL-EVs from unstimulated CLL cells (blue line). Dotted lines are the respective isotype control experiments. The bar chart shows changes to MFI expression of CD69 following treatment with CLL-EVs (Student  $t$  test,  $* P<0.05$ ) ( $n=4$ ).

**Figure 3. EVs from CD40/IL-4-stimulated CLL cells rapidly enter autologous CD4<sup>+</sup> T cells.**

CD4<sup>+</sup> T cells from CLL patients were incubated with fluorescently PKH67-labeled CD40/IL-4 CLL-EVs for the indicated times before washing and fixation (0 time-point, 24 and 48 hours (h)). EV transfer studies were followed by confocal imaging. **(A)** Quantification of EV internalization using ImageJ software. MFI of PKH67-EVs  $\pm$  SEM ( $n=3$ ).  $**P<.01$ . **(B)** The confocal images show maximal uptake of PKH67-labeled CLL-EVs into autologous CD4<sup>+</sup> T-cells by 24 h (CD4<sup>+</sup> T cell F-actin, rhodamine phalloidin (red), PKH67-labeled CD40/IL-4 CLL-EVs (green), CD4<sup>+</sup> T cell nuclei (blue) and a merged image (bottom right of image panels). Scale bar 20  $\mu$ m. **(C)** Confocal images showing serial sections of confocal micrographs (CD4<sup>+</sup> T cell F-actin, rhodamine phalloidin (red) and PKH67-labeled CD40/IL-4 CLL-EVs (green)) showing internalization of CLL-EVs within CD4<sup>+</sup> T cells by 24 h. Scale bar 5  $\mu$ m.

**Figure 4. CD4<sup>+</sup> T cells exposed to autologous EVs from CD40/IL-4 stimulated CLL cells exhibit enhanced immunological synapse signaling and proliferation. (A)** Confocal images of T cell F-actin immune synapse formation (F-actin rhodamine phalloidin, red) interactions with



autologous tumor CLL cells (CMAC-labeled, blue) from a representative patient. CD4<sup>+</sup> T cells (negatively selected) were treated with purified CLL-EVs for 48 hours (CLL-EVs following CD40/IL-4 stimulation (CD40/IL-4 CLL-EVs) compared to control EVs from unstimulated CLL cells (CLL-EVs). Treated T cells were then conjugated with autologous CLL B cells (pulsed with superantigen, sAg cocktail and labeled with CellTracker blue dye, CMAC). T cell conjugates were analyzed by immunofluorescence and confocal microscopy. Confocal images (n = 10 per patient treatment sample) were acquired using an A1R confocal microscope (Nikon) and analyzed with NIS-Elements software (Nikon). Original magnification 60x. Scale bar 5  $\mu$ m. Quantitative image analysis measured the total area ( $\mu$ m<sup>2</sup>) of F-actin polymerization (F-actin rhodamine phalloidin, red) at CD4<sup>+</sup> T cell contact sites and synapses with CLL cells within the experimental T cell population. The dot plot shows the mean synapse/interaction area ( $\mu$ m<sup>2</sup>)  $\pm$  SEM from a representative patient (from 6 independent CLL samples) (Mann-Whitney t-test, \*\**P* < .01). (B) Intercellular CD4<sup>+</sup> T cell: tumor CLL conjugates formed between CD40/IL-4 CLL-EVs or CLL-EVs treated CD4<sup>+</sup> T cells and sAg-pulsed autologous CLL tumor cells were scored using a confocal microscope (scale bar 5  $\mu$ m) and image analysis software. Bar chart shows the mean % CD4<sup>+</sup> T cell: tumor CLL conjugates  $\pm$  SEM from 6 independent CLL samples with 100 random T cells analyzed per experiment (Mann-Whitney t-test, \**P* < .05). (C) CD4<sup>+</sup> T cell: tumor CLL conjugates formed during 20 min were fixed, permeabilized and stained with anti-Lck (green). Tumor cells were labeled with CellTracker blue dye, CMAC. T cells are denoted by white dashed lines. Quantitative image analysis (relative recruitment index, RRI) of Lck accumulation at the immunological synapse is shown, representative evaluation of 20 conjugates from 3 independent experiments, with the mean value shown as a blue bar  $\pm$  SEM. Scale bar 5  $\mu$ m. (D) MFI was calculated for CD4<sup>+</sup> T cell CD69 expression levels (Alexa 488, green fluorescent channel) following treatment with CD40/IL-4 CLL-EVs or CLL-EVs. T cells are denoted by white dashed lines. Tumor cells were labeled with CellTracker blue dye, CMAC. Dot plot chart shows the CD69 MFI  $\pm$  SEM for a representative patient from 6 CLL samples (Mann-Whitney t-test, \*\**P* < .01). Original magnification 60x. Scale bar 5  $\mu$ m. (E) Nuclear Ki-67 expression, a marker for cellular proliferation, was measured in autologous patient CD4<sup>+</sup> T cells exposed to CLL-EVs (CD40/IL-4 CLL-EVs or CLL-EVs) and with and without anti-CD3/-CD28 crosslinking stimulation for 72 hours (-CD3/-CD28 and unstim. respectively). Bar chart shows mean Ki-67  $\pm$  SEM for 3 independent CLL patient experiments (one way ANOVA, \*\**P* < .01). (F) The confocal images show increased nuclear (DAPI, blue) Ki-67 expression (Alexa-647 denoted using green with analysis software) in CD4<sup>+</sup> T cells (F-actin, red) treated with CD40/IL-4 CLL-EVs compared with co-culture with control CLL-EVs. Scale bar 5  $\mu$ m. (G) Z-stack confocal analysis (3-D central T cell region analysis) reveals increased nuclear (DAPI, blue) Ki-67 expression (Alexa-647 denoted using green with analysis software) in CD4<sup>+</sup> T cells (F-actin, red) treated with CD40/IL-4 CLL-EVs compared with control CLL-EVs. Scale bar 5  $\mu$ m.

**Figure 5. CD4<sup>+</sup> T cells exposed to autologous EVs from CD40/IL-4 stimulated CLL cells exhibit enhanced CCL19-induced migration.** Primary patient CD4<sup>+</sup> T cells were treated with



autologous CLL-EVs for 48 hours (CLL-EVs following CD40/IL-4 stimulation, (CD40/IL-4 CLL-EVs) compared to control EVs from unstimulated CLL cells (CLL-EVs)). Treated T cells were then allowed to adhere to immobilized ICAM-1 for 10 minutes following exposure to the chemokine CCL19. Video microscopy (Biostation IM, Nikon) was used to observe the migration of CD4<sup>+</sup> T cells for 20 minutes before the migration of individual cells was tracked (NIS-Elements analysis software, Nikon) (A) Representative phase-contrast images (original magnification x20) and motility tracks (colored) of CD4<sup>+</sup> T cells from CLL patients treated with experimental CLL-EVs as denoted (upper microscopy images). Representative migratory tracks of individual T cells from CLL patients treated with CLL-EVs are shown in the lower data plots (n = 20 cells per experiment). Dot plot charts show the (B) speed of migration ( $\mu\text{m}/\text{min}$ ) and (C) distance of migration ( $\mu\text{m}$ ) for a representative patient from 6 independent CLL samples (Mann-Whitney t-test,  $**P < .01$ ).

**Figure 6. Knockdown of miR-363 in parental CLL cells prevents the ability of released CLL-EVs to confer altered functional properties to autologous CD4<sup>+</sup> T cells.**

MiR-363 was knocked down in CLL cells using LNA-based antisense oligonucleotides, prior to CD40/IL-4 stimulation and isolation of released EVs (anti-miR-363 compared to scrambled control (Con.) antisense miR oligonucleotides). CD4<sup>+</sup> T cells (negatively selected) were treated with purified CLL-EVs from the transfected autologous parental CLL cells. Treated T cells were then conjugated with autologous CLL B cells (pulsed with superantigen, sAg cocktail and labeled with CellTracker blue dye, CMAC). T cell conjugates were analyzed by immunofluorescence and confocal microscopy as described in Figure 4. Confocal images (n = 10 per patient treatment sample) were acquired using an A1R confocal microscope (Nikon) and analyzed with NIS-Elements software (Nikon). Original magnification 60x. Scale bar 5  $\mu\text{m}$ . Dot plots charts show (A) the mean F-actin polymerization (rhodamine phalloidin, red) synapse/interaction area ( $\mu\text{m}^2$ )  $\pm$  SEM from a representative patient (from 3 independent CLL samples), (B) the mean RRI of Lck accumulation (green) at the immunological synapse, representative analysis ( $\pm$  SEM) from 3 independent patient experiments and (C) the CD69 MFI  $\pm$  SEM for a representative patient from 3 independent CLL patient samples. CD69 expression is denoted in white. Treatment of autologous CD4<sup>+</sup> T cells with CLL-EVs derived from miR-363 knocked down cells (anti-miR-363) blocked modulation of T cell migration rates when compared to EVs derived from control transfected tumor cells (Scrambled Con. miR). Dot plot charts show the (D) speed of migration ( $\mu\text{m}/\text{min}$ ) and (E) distance of migration ( $\mu\text{m}$ ) for a representative patient from 3 independent CLL samples. (Mann-Whitney t-test, ,  $*P < .05$ ,  $**P < .01$ , n.s. not significant).

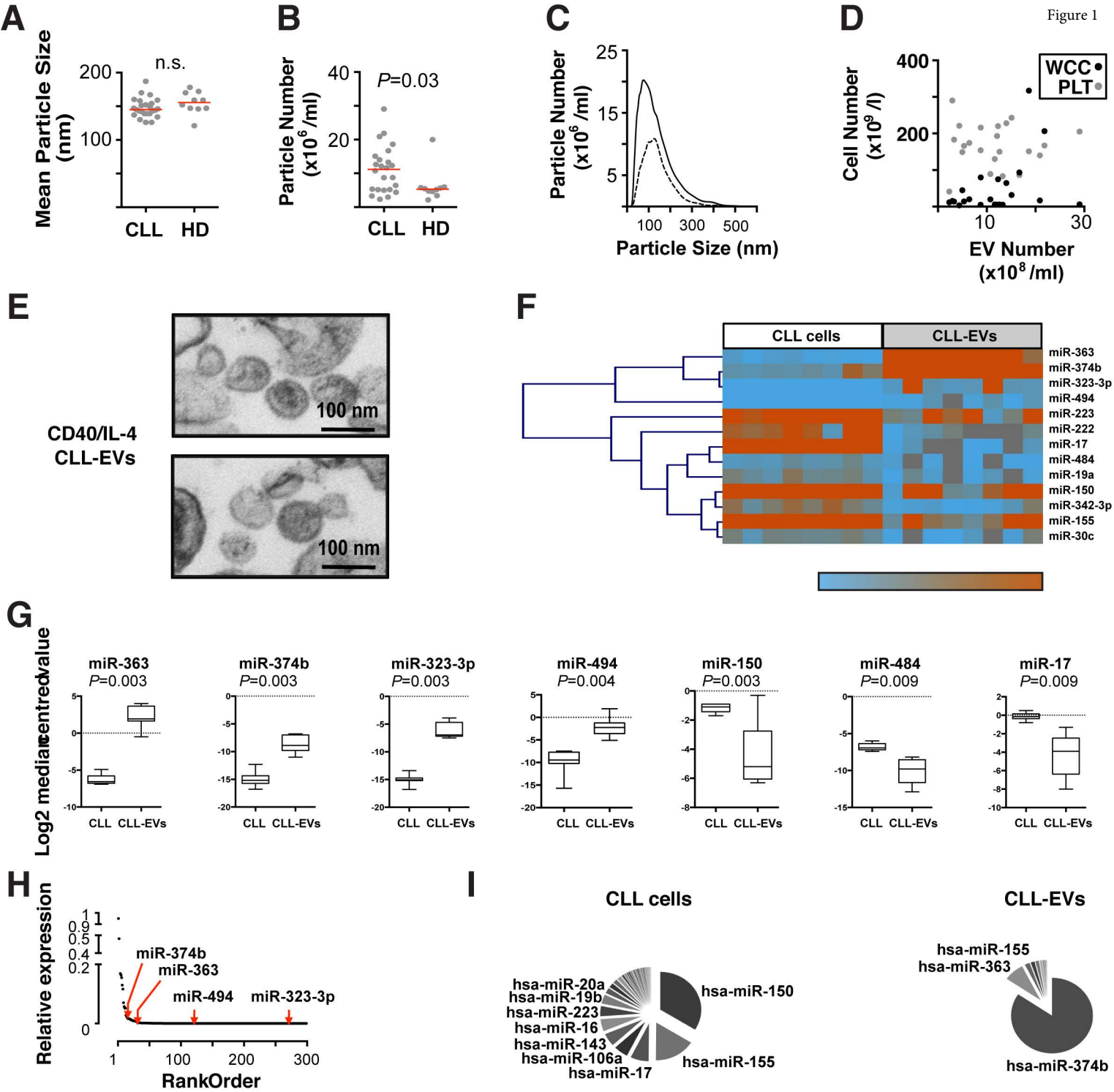
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**A**

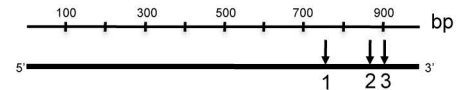
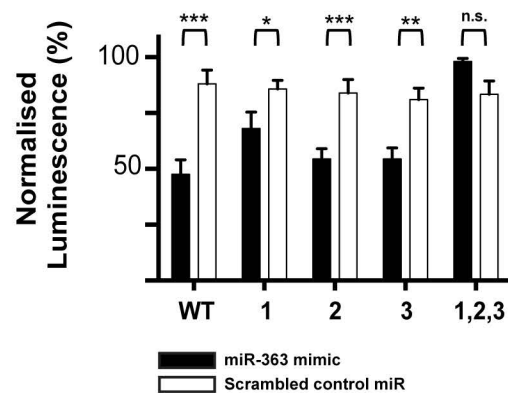
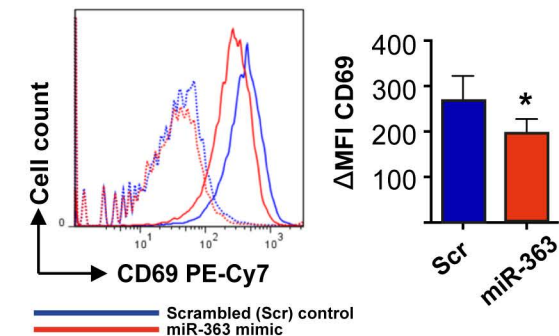
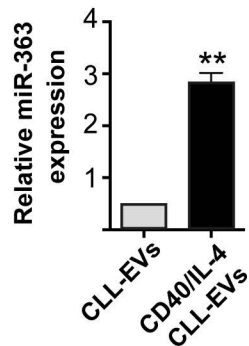
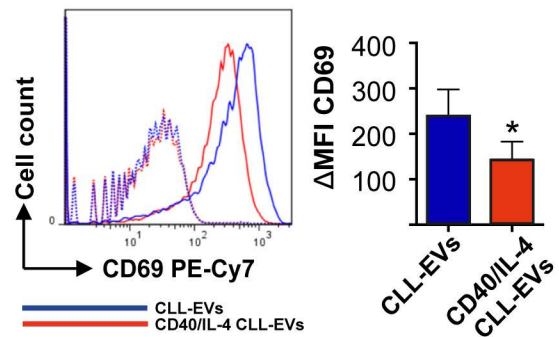
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**B**

Figure 2

**C****D****E****F****G**



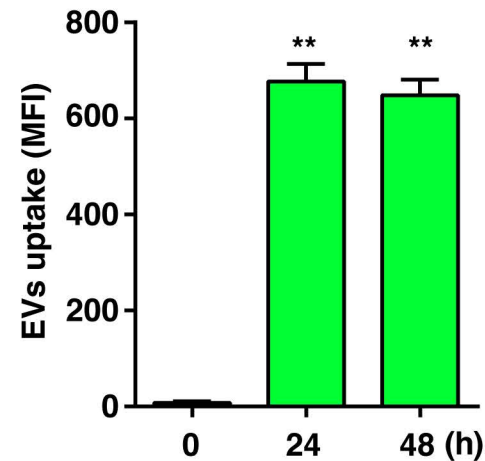
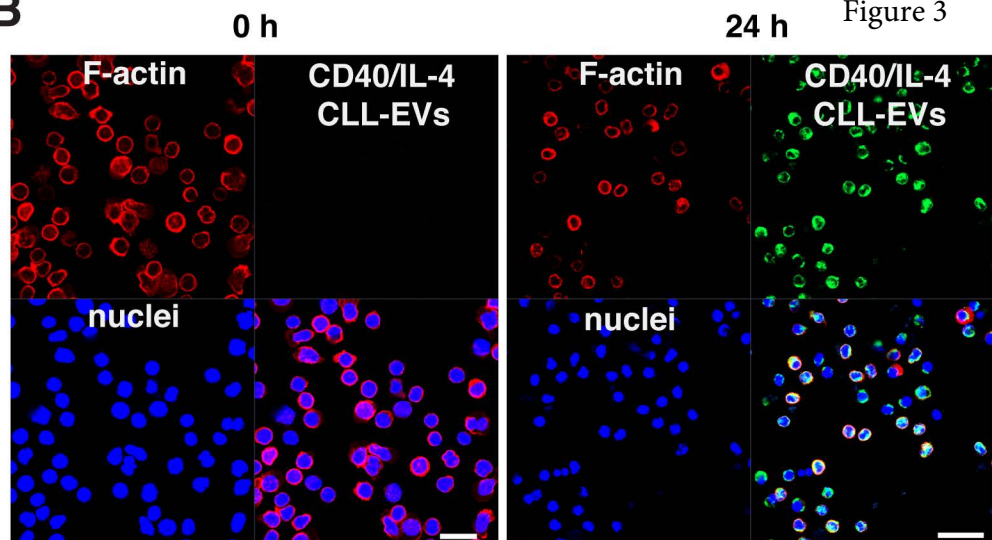
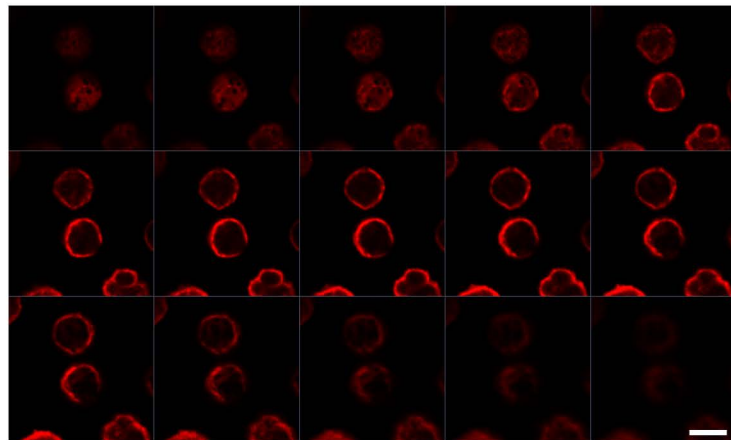
**A****B**

Figure 3

**C**

0 h - serial sections (F-actin, CLL-EVs)



24 h - serial sections (F-actin, CLL-EVs)

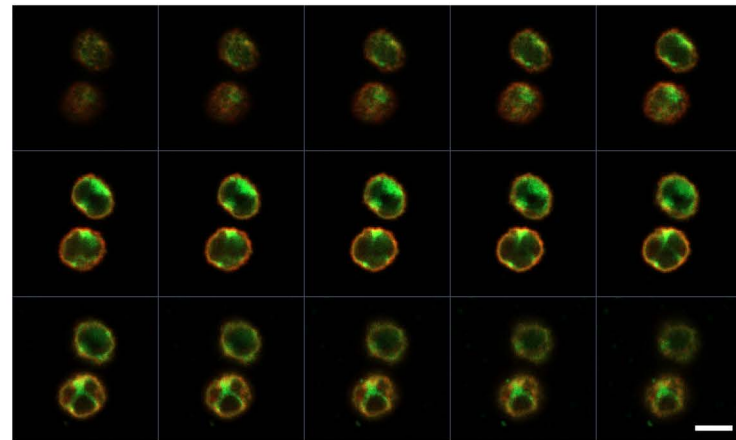
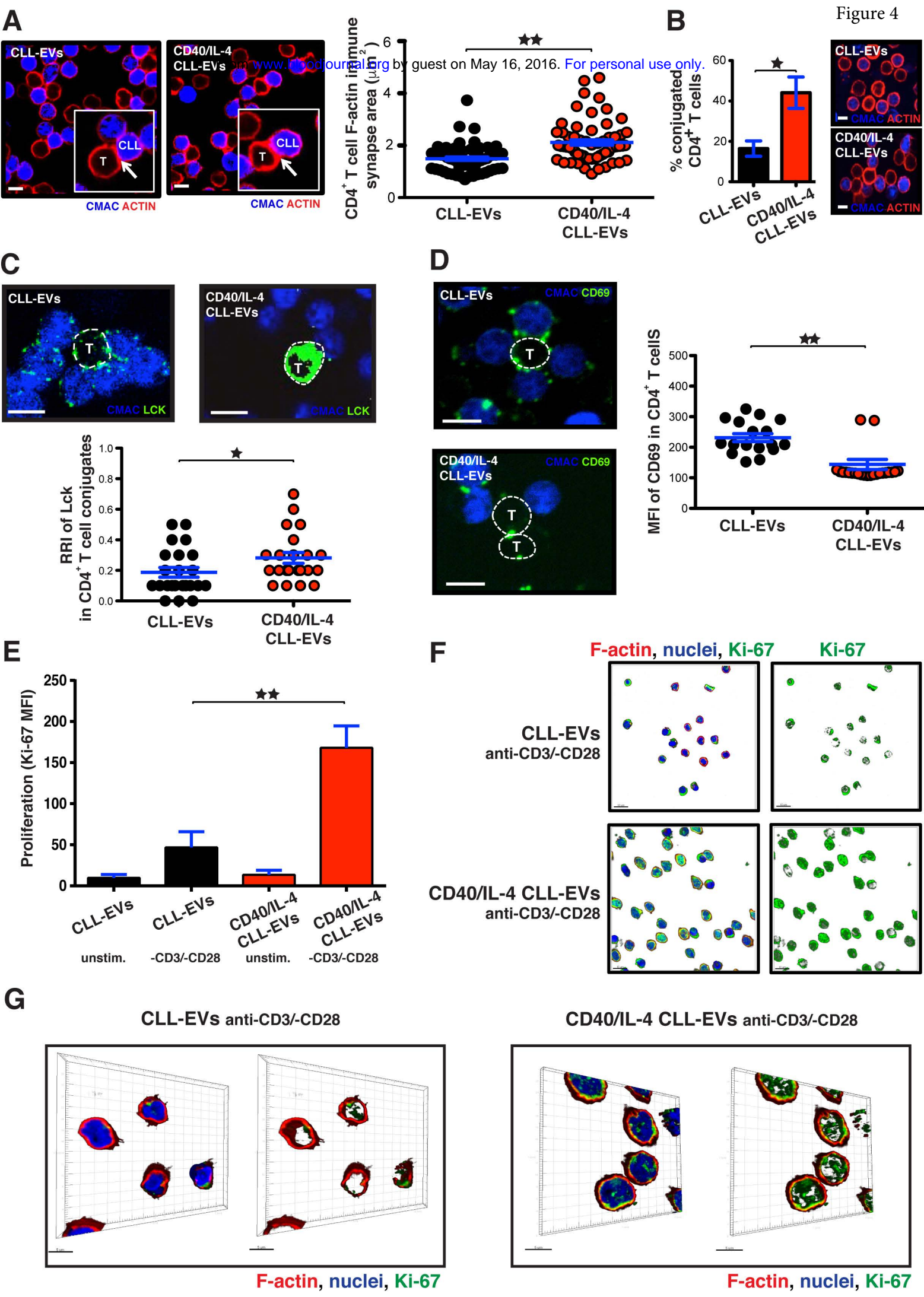
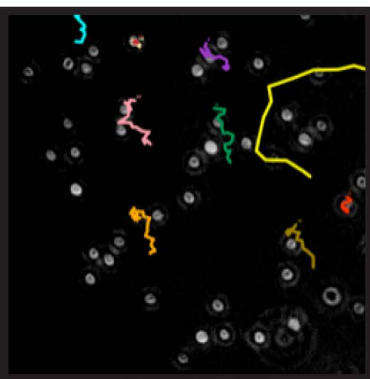
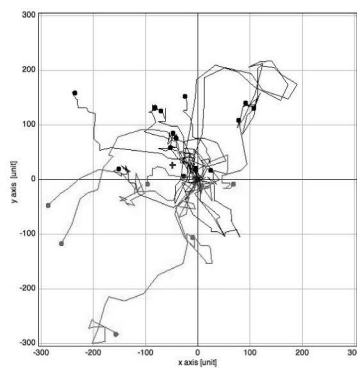
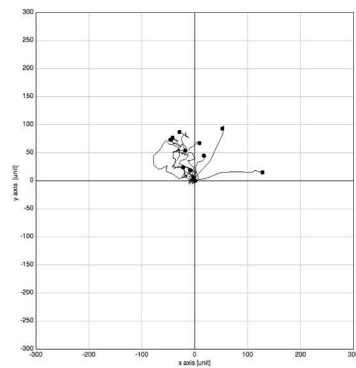
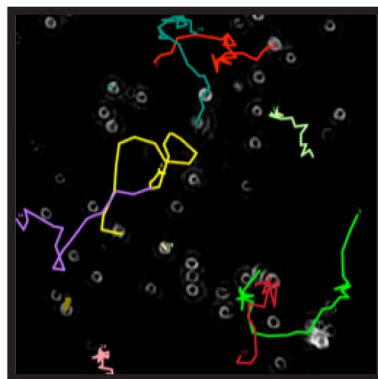
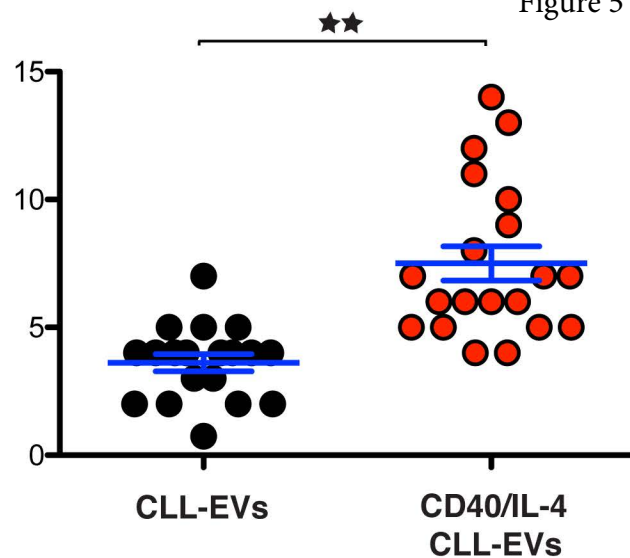
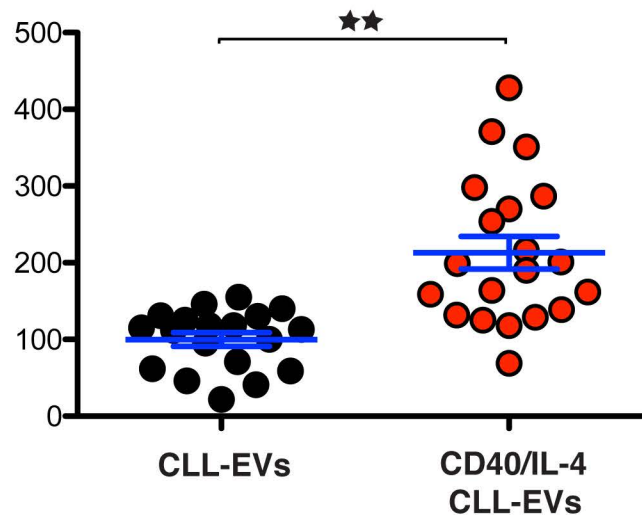


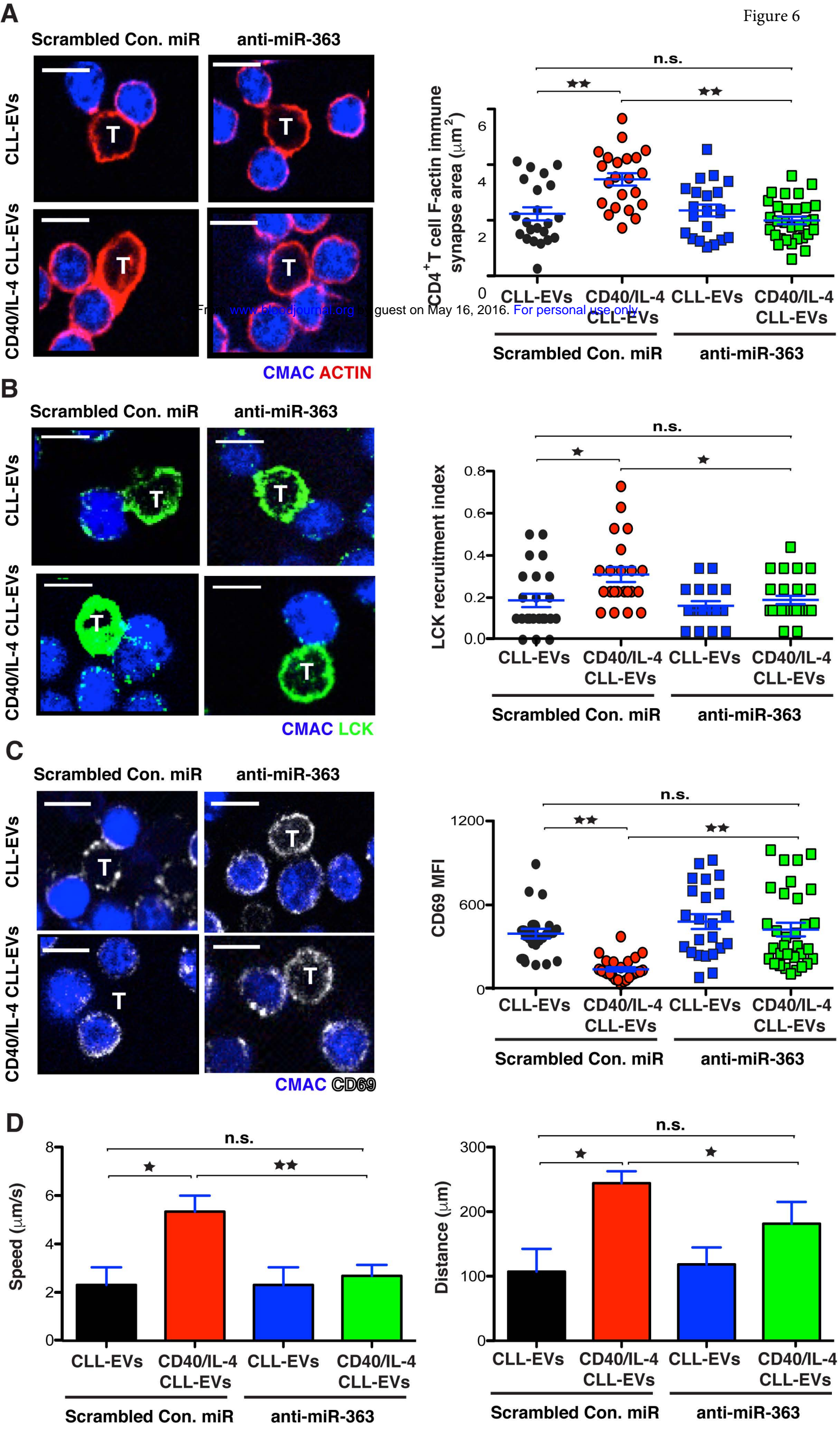


Figure 4



**A****CLL-EVs****CD40/IL-4  
CLL-EVs****B****Speed of migration  
CD4<sup>+</sup> T cell (μm/min)****C****Distance of migration  
CD4<sup>+</sup> T cell (μm)**







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## **Extracellular vesicles released by CD40/IL-4 stimulated chronic lymphocytic leukemia cells confer altered functional properties to CD4<sup>+</sup> T cells**

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